Regulation of Hypoxanthine DNA Glycosylase in Normal Human and Bloom’s Syndrome Fibroblasts

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ABSTRACT

The regulation of the base excision repair enzyme hypoxanthine DNA glycosylase was examined in normal human skin fibroblasts (NHS) and fibroblasts from a patient with Bloom’s syndrome. Using randomly proliferating cells and those synchronized at specific intervals in the cell cycle, enzyme levels were shown to become elevated several fold in a proliferation-associated manner. In NHS synchronized in G0 by serum deprivation or in G1 by isoleucine deprivation, maximal enzyme levels were reached prior to maximal rates of DNA synthesis. In Bloom’s syndrome cells synchronized in this manner, these two activities were coincident. Cells synchronized at the G1-S border by hydroxyurea exhibit an initial wave of DNA synthesis upon removal of the drug. The cells then undergo another DNA synthetic cycle climaxing 18–21 h after release. Maximal hypoxanthine glycosylase activity of hydroxyurea-synchronized Bloom’s cells was observed during the second round of DNA synthesis. However, in NHS the peak of enzyme activity was observed as early as 9 h prior to the second round of DNA synthesis. To determine if hypoxanthine glycosylase could be induced in the absence of DNA synthesis, serum-synchronized NHS were released in the presence of hydroxyurea. The results showed that inhibition of DNA synthesis did not diminish glycosylase induction which demonstrated that DNA replication was not required for glycosylase induction.

INTRODUCTION

Numerous studies have established the relationship between the initiation of neoplasia and the presence of chemical- or radiation-induced DNA damage. DNA-carcinogen adducts such as O6-alkylguanine or O4-alkylthymidine are promutagenic and result in GC to AT transitions (1). Further, spontaneous deamination of cytidine to uracil and adenine to hypoxanthine represents mutational alterations in DNA. Enzymes which remove these lesions have been identified in mammalian cells (2–4). For the most part, their specific activities in diverse tissues correlate inversely with the susceptibility of such tissues to the action of carcinogenic agents.

Eukaryotic cells specifically regulate the activities of diverse DNA replicative enzymes during cell proliferation. In some instances, enzyme activities are induced to supply the increased quantities of metabolic intermediates for DNA synthesis. Thus, dihydrofolate reductase, thymidine synthase, and thymidine kinase activities are increased as a function of growth (5–7). In contrast, in other instances, DNA replication and subsequent cell division would require the diminution of specific DNA metabolic enzymes; thus TTP dephosphorylase activity is diminished during cell proliferation (8). In each instance, the regulation of each gene would be an essential requirement for DNA synthesis.

Similarly, the quantity of DNA repair enzymes in human cells appears to be dependent on the proliferative state of the cell. Previous studies described the relationship between the increase in the activity of base excision repair enzyme activities to the induction of DNA replication and to cell division (9–11).

In base excision repair, the initial enzymatic step is performed by a DNA glycosylase, which catalyzes the hydrolysis of the glycosyl bond releasing the modified base (2–4). Subsequent nuclease and DNA polymerase activities cleave and extend the resultant apurinic or apyrimidinic site in DNA and resynthesize the appropriate nucleotide sequences. Lastly, DNA ligase re-establishes the covalent linkage of the repaired DNA.

In normal human cells, uracil DNA glycosylase and the base excision repair pathway were enhanced prior to the induction of DNA replication and DNA polymerase in serum-stimulated cells (12, 13). We suggested that this enhancement of repair enzyme activity might serve as a proofreading or prescreening mechanism to help prevent the fixation of mutagenic alterations in the genome (14). Recently, we reported that hypermutable cells from Bloom’s syndrome patients (15, 16) were characterized by an inability to correctly regulate the proliferation-dependent enhancement of base excision repair (17). In particular, uracil DNA glycosylase and the base excision repair pathway were enhanced coordinately with DNA replication. This would suggest that these individuals might be unable to efficiently repair carcinogen-modified DNA prior to replication. To examine the specificity of this alteration in human gene expression we examined whether the regulation of another base excision repair enzyme, hypoxanthine DNA glycosylase, showed similar alterations in Bloom’s syndrome cells. This enzyme excises hypoxanthine residues from DNA produced by the mutagenic deamination of adenine (18). We now report that normal human cells enhanced this base excision repair enzyme prior to DNA replication. However, in Bloom’s syndrome cells, there was a temporal alteration such that the enzyme was induced concomitantly with DNA replication. This altered temporal sequence was identical to that previously observed with the uracil DNA glycosylase. These results suggest that there is an intrinsic genetic defect in Bloom’s syndrome cells which results in the altered regulation of at least two individual base excision repair enzymes during cell proliferation.

MATERIALS AND METHODS

Cell Culture. Normal human skin fibroblasts (CRL 1222; American Type Culture Collection, Rockville, MD) and Bloom’s syndrome fibroblasts (GM 2548; NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) were obtained at passage 10 or lower. Cells were propagated at 37°C in 5% CO2 in air in 150-cm2 tissue culture flasks in Dulbecco’s minimal essential medium (Gibco) containing glutamine, antibiotics, and 10% fetal bovine serum unless specified. Cells were subcultured after attaining confluence but were not maintained beyond passage 15.

Methods of Cell Synchronization. Cells were synchronized in early G1 by serum, middle G1 by isoleucine deprivation, or at the G1-S border by incubation with 0.5 mM HU.1 Cells to be synchronized by serum

1 The abbreviations used are: HU, hydroxyurea; NHS, normal human skin fibroblasts; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography.

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deprivation were seeded into 100 mm plastic dishes at a density of 10^6 cells per dish in DMEM containing 0.3% serum. After 5 days in culture, cells were released into the cell cycle by the addition of 20% serum containing medium. Synchronization by isoleucine deprivation was accomplished by seeding cells as above in 10% dialyzed fetal bovine serum containing DMEM without isoleucine (Gibco). After 3 days in culture, cells were released by the readdition of complete DMEM. HU synchronization of cultures was accomplished by the addition of a stock solution of HU to a final concentration of 0.5 mm. After 18 h cells were rinsed twice with prewarmed Hanks’ balanced salt solution (Gibco) and incubated with DMEM. For some experiments cells were given 2 mm HU immediately after release.

For asynchronous growth, confluent cells in 150-cm² tissue culture flasks were trypsinized and seeded in 100 mm dishes at a density of 1–2 x 10^6 cells/dish. Cells were grown for 6 days without medium change.

Cell Extracts. Cells to be used for various assays were rinsed twice with cold Dulbecco’s phosphate-buffered saline (Gibco) and harvested with a rubber policeman. After sedimentation at 300 x g, cell pellets were frozen at -20°C. Prior to assay, frozen cell pellets were resuspended in 1 ml of 20 mM Tris-HCl (pH 8.0)-1 mM DTT-20% (v/v) glycerol and sonicated at 70 W for 30 s at 0°C, with a Bransonic sonicator equipped with a needle probe. Extracts were centrifuged at 300 x g to remove unbroken cells.

Preparation of Hypoxanthine DNA Glycosylase Substrate. Hypoxanthine DNA glycosylase was assayed using a substrate prepared by a modification of a method reported by Karran (19). [3H]dATP (Amersham or ICN; specific activity, 26 Ci/mm, 5.0 mCi) was deaminated for 5 h with freshly prepared nitrous acid (50% ethanol-50% 1.6 N ammonium hydroxide). HPLC analysis demonstrated that the product was 90% [3H]dITP. [3H]dITP was incorporated into poly dC oligo dG12-m (PL Biochemicals) using Escherichia coli DNA polymerase I. A 10-ml extraction, and the phenol was removed by dialysis as previously described (8).

Assay of Hypoxanthine DNA Glycosylase. Hypoxanthine DNA glycosylase activity was assayed in the presence of approximately 1.5 μg of poly(dG)·poly [3H](dI), 100 μM Tris-HCl, 1 mM EDTA, 0.1 mM DTT, and 20% (v/v) glycerol in a volume of 100 μl. After incubation at 37°C for 120 min, the reaction mix was precipitated with ethanol. Ethanol-soluble material was concentrated under nitrogen. Authentic hypoxanthine was added, and the sample was injected directly on a Waters C8-Bondapak reversed-phase column eluted isocratically at 1 ml/min with 10 mM ammonium formate, pH 3.8. Fractions were collected at 30-s intervals. The arrow indicates the position of the hypoxanthine marker. Inset, release of hypoxanthine-specific radioactivity as a function of time of incubation (A, 10 μl of substrate and 10 μg of cell protein) or quantity of cell protein in assay mixture (B, 10 μl of substrate, 10 μg of protein, incubation for 120 min at 37°C).

RESULTS

Product Analysis and Characterization of Hypoxanthine DNA Glycosylase Reaction. HPLC analysis of the ethanol-soluble fraction after the glycosylase assay revealed the presence of 3H products aside from hypoxanthine (Fig. 1). Although uracil DNA glycosylase can be examined by simply quantitating ethanol-soluble radioactivity (9), the presence of other products necessitated separation of authentic hypoxanthine in each assay. Therefore, 1–2 μg of hypoxanthine was used as a marker in each HPLC analysis. This assured that only hypoxanthine released by enzymatic activity was quantitated. The initial peak of radioactivity which was not retained by the column was observed in reactions which did not contain any cell extract. Assay conditions were established by examining the effect of incubation time and quantity of cell protein on the release of [3H]hypoxanthine from the substrate (Fig. 1, inset). Hypoxanthine release was linear for up to 150 min of incubation at 37°C in reactions which contained 20 μg of cell extract protein. Further, enzyme activity was proportional to the amount of cell extract added (results not shown). Glycosylase assays of all cell extracts were routinely incubated for 120 min and were performed with an 8-fold range of cell protein.

Induction of Hypoxanthine DNA Glycosylase. To determine if the elevation in the levels of this base excision repair enzyme was a process associated with cell proliferation, confluent NHS and Bloom’s syndrome cells were subcultured at a lower density and allowed to grow over a 5-day interval. Prior to harvest, cultures were pulsed with tritiated thymidine to quantitate DNA synthesis. The results are shown in Fig. 2. In each cell type, the rate of DNA synthesis peaked at Day 1 and then declined throughout the rest of the growth period. Total DNA synthesis continued, and cell numbers increased steadily through Days 4 and 5 (not shown) after which the cultures became confluent. The specific activity of hypoxanthine DNA glycosylase increased 2-fold in NHS and 3-fold in Bloom’s cells. Maximal induction was observed during Days 2–4 of the growth period. Enzyme levels then declined thereafter. NHS and Bloom’s syndrome cells demonstrated equivalent levels of enzyme induction and regulated these levels similarly during this time period. In these experiments, DNA synthesis had nearly returned to basal levels while enzyme activity was almost maximal.

Regulation of Hypoxanthine DNA Glycosylase in Synchronized NHS. To determine if hypoxanthine glycosylase regulation was an authentic cell cycle-mediated event, cells were synchronized at distinct stages of the cell cycle. After release from G0 by the readdition of serum, the induction of DNA synthesis and glycosylase were monitored. As shown in Fig. 3A,
HYPOXANTHINE DNA GLYCOSYLASE REGULATION

Fig. 2. Induction of hypoxanthine DNA glycosylase and DNA synthesis in NHS (A) and Bloom's syndrome cells (B) after release from confluence inhibition. Confluent cells were seeded at 1–2 × 10⁶ cells/plate and allowed to proliferate without medium change. At 24-h intervals, cells were pulsed for 30 min with [³H]thymidine prior to collection. The average enzyme activity over an 8-fold range of cell protein is expressed as pmol of hypoxanthine released per mg of cell protein. All figures represent typical experiments. The absolute numbers representing DNA synthesis and hypoxanthine DNA glycosylase activities in each cell synchrony experiment should not be compared, as each individual experiment described in A to C, respectively, was performed several mo apart. However, within each individual experiment, all reaction parameters and enzyme activities were reproducible.

serum-synchronized NHS displayed maximal levels of DNA synthesis about 24–27 h after release. Hypoxanthine DNA glycosylase levels were induced 3.7-fold at 18 h after cell stimulation. Thereafter both DNA synthesis and enzyme activity diminished.

Isoleucine deprivation was used to synchronize cells in middle G₁. Upon release into the cell cycle, maximal DNA synthesis was observed at 18 h (Fig. 3B). The regulation of hypoxanthine glycosylase levels was altered in response to the change in timing of DNA synthesis. Enzyme activity was induced over 2-fold and was maximal 15 h after release from the G₁ block. Thus, the temporal relationship between DNA synthesis and enzyme induction was not characteristic of the method used to achieve cell synchrony but would seem to be dependent on factors regulating DNA synthesis.

NHS were synchronized at the G₁-S juncture by incubation with 0.5 mM HU. Upon removal of the drug, an initial induction of DNA synthesis was detected (Fig. 3C). In two experiments, this first S phase occurred 3 h after HU removal. Little change in enzyme levels was associated with this early peak. However, enzyme activity was slightly elevated as compared to that observed prior to HU addition. In the next cell cycle maximal DNA synthesis was detected at 21 h. NHS exhibited a peak of glycosylase activity at the 9-h time point, considerably earlier than the peaks of activity detected in serum- or isoleucine-synchronized cells. Glycosylase activity then declined to levels observed prior to the addition of HU (results not shown).

Regulation of Hypoxanthine DNA Glycosylase in Bloom’s Syndrome Cells. To examine whether the temporal sequence of glycosylase regulation was altered in hypermutable Bloom’s syndrome cells, synchronization experiments similar to those described for NHS were performed (Fig. 4). In G₀ synchronized cells DNA synthesis was maximal 24 h after serum stimulation (Fig. 4A). Hypoxanthine DNA glycosylase activity also peaked at this time. Glycosylase specific activity was increased 4-fold concomitantly with maximal DNA synthesis. This fundamental difference in the timing of these two activities as compared to NHS was reproducible and characteristic of Bloom’s syndrome cells.

Fig. 3. Regulation of hypoxanthine glycosylase induction in NHS. NHS were plated and synchronized as described in the text by serum deprivation (A), isoleucine deprivation (B), or inclusion of 0.5 mM hydroxyurea in the medium (C). Cells were released into the cell cycle by the readdition of complete medium or removal of drug. At 3-h intervals, cells were pulsed with [³H]thymidine and harvested. DNA synthesis and enzyme activity were quantitated using the same cell extracts.
To examine the relationship between glycosylase regulation and DNA replication in cells synchronized at the G1-S border, Bloom's syndrome fibroblasts were incubated in 0.5 mM HU (Fig. 4C). Hypoxanthine glycosylase induction in HU-synchronized Bloom's cells differed greatly from that observed in NHS. Bloom's syndrome cells demonstrated the same initial wave of DNA synthesis 3 h after the removal of HU as did NHS. Incorporation of [3H]thymidine attained 45,000 cpm/mg of protein. The next synthetic cycle was at 21 h, as in NHS. Thus the regulation of DNA synthesis in NHS and Bloom's cells was similar. However, in two experiments with Bloom's cells maximal glycosylase induction occurred later than that observed for NHS. This is in contrast to the very early induction of hypoxanthine glycosylase in HU-synchronized NHS (Fig. 3C).

The experiments reported thus far have focused on the temporal relationship between DNA synthesis and enzyme induction. However, we wanted to determine if the induction of DNA synthesis was required for the enhancement of hypoxanthine DNA glycosylase. Therefore, serum-synchronized NHS were stimulated to reenter the cell cycle in the presence of HU. In control cultures stimulated with serum in the absence of HU, DNA synthesis was maximal at 21 h (Fig. 5A). Hypoxanthine glycosylase activity was induced about 3-fold and was maximal 6 h prior to maximal DNA synthesis.

Cells were then allowed to enter the cell cycle and were given HU 9 h after serum stimulation. At this time, DNA synthesis is still not detected (Fig. 5A). However, cells presumably are committed to synthesize DNA and can synthesize deoxyribonucleotides. In this experiment, hypoxanthine DNA glycosylase was induced in the absence of DNA synthesis (Fig. 5B).

We then wanted to determine if an early, hydroxyurea-sensitive event affected the regulation of hypoxanthine glycosylase. When 2 mM HU was present upon serum stimulation (Fig. 5B), DNA synthesis was inhibited by over 75%. Enzyme induction in these cultures was unaffected and increased about 3-fold above basal levels. Thus neither the synthesis of deoxyribonucleotides nor DNA replication itself significantly affected the induction of glycosylase activity.

**DISCUSSION**

The induction and regulation of the base excision repair enzyme hypoxanthine DNA glycosylase were examined in normal human cells and in hypermutable Bloom's syndrome fibroblasts. In both cell strains, initial experiments determined that this DNA repair enzyme was induced as a function of cell proliferation. The 3-fold induction of the hypoxanthine DNA glycosylase is in accord with similar proliferative dependent increases of a variety of eukaryotic enzymes. In contrast, in prokaryotic systems, many DNA repair enzyme activities may increase by 30-fold as part of an induction process (21, 22). A more precise examination of hypoxanthine DNA glycosylase regulation in NHS and in Bloom's syndrome cells was performed with cells synchronized at discrete stages in the cell cycle. In NHS, the glycosylase was consistently induced prior to the induction of DNA synthesis independently of both the
synchronization method and the stage at which the cells were synchronized. In Bloom's syndrome cells, the glycosylase and DNA synthesis were simultaneously induced in each experiment. This would suggest that this aberration of repair regulation in Bloom's syndrome cells is intrinsic to that human genetic syndrome.

Two systems may be utilized to examine the regulation of cellular processes as a function of growth. (a) Alterations in biochemical events which occur due to the activation of quiescent cells to a proliferative mode can be quantitated after a mitogenic stimulus. Thus, previous results demonstrated that eukaryotic cells enhance the specific activities of DNA repair enzymes (23–27), the levels of DNA excision repair synthesis (28–32), and the excision rates of DNA adducts (33, 34) during this transition state. In this paper, we demonstrated that proliferation of normal human and in Bloom's syndrome cells resulted in increases in hypoxanthine DNA glycosylase activity. (b) Selective gene expression intrinsic to the cell cycle may be examined by analysis of synchronized cells or by the physical isolation of cells at discrete stages of the cell cycle. In this paper, human cells were synchronized in G₀ by serum, in G₁ by isoleucine, and at the G₁–S border by hydroxyurea. These procedures allowed a more precise examination of hypoxanthine DNA glycosylase regulation by the manipulation of the degree to which glycosylase induction had progressed up to that point in the cell cycle. In addition, the continual alteration in the timing of DNA synthesis demonstrated the strict temporal relationship between glycosylase induction and DNA replication. Further, the use of several synchronization methods would argue against the possibility that results which were obtained were dependent on the particular method of synchronization. In normal human cells, enzyme activity was increased immediately prior to maximal DNA synthesis in either serum- or isoleucine-synchronized cells. The greater temporal separation of these two cellular processes was detected in HU-synchronized cells.

To begin to examine the signals which may control repair regulation in relation to DNA replication, the induction of hypoxanthine DNA glycosylase in serum-stimulated cells was examined after hydroxyurea exposure. Our initial evidence suggested the independence of glycosylase induction prior to the initiation of DNA synthesis. Inhibition of DNA synthesis in serum-synchronized cells by HU at the time of release from the serum block did not diminish the enhancement of glycosylase activity. Similarly, no significant alteration was detected in the temporal sequence of glycosylase induction. Thus, the regulatory signal which initiates glycosylase induction would be expressed at an earlier interval. Further, the ability to uncouple DNA synthesis from enzyme stimulation shows that the former is not required for the latter.

Several human genetic syndromes which predispose to malignancy may be characterized by cellular hypersensitivities to DNA damaging agents as well as deficiencies in repair of lesions produced by these agents (35). Although these cellular defects are well characterized, the molecular mechanisms which underlie such deficiencies remain unknown. For that reason, we began to investigate whether such cells might be deficient in their proliferative dependent regulation of specific DNA repair pathways. In particular, we demonstrated that hypermutable Bloom's syndrome cells were characterized by an inability to enhance the uracil DNA glycosylase prior to DNA synthesis during cell stimulation (17). In this paper, we demonstrate that these cells fail to regulate hypoxanthine DNA glycosylase properly as well. Further, the alteration in hypoxanthine DNA glycosylase regulation was identical to that observed for the uracil DNA glycosylase. Thus, the alteration in uracil DNA glycosylase regulation in Bloom's syndrome cells was not unique but may represent a general defect in the proliferation-dependent regulation of base excision repair genes. Future work at the molecular level is required to elucidate the mechanisms of the coordinate regulation of base repair excision repair in normal human cells and what common alteration may exist in hypermutable cells from cancer-prone Bloom's syndrome patients.

REFERENCES


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